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Nigrospora macarangae associated with *Ficus pumila* leaf spots in Guangdong Province, China

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Abstract

Ficus pumila is a perennial vine in the *Moraceae*. It is a plant with high medicinal and edible value. In this study, *Ficus pumila* samples were collected in Guangzhou, Guangdong Province, China, exhibiting typical leaf spot symptoms. Through tissue isolation, we obtained fungal strains and identified them using morphological characteristics and phylogenetic methods. Phylogenetic analysis was performed using sequence data for the internal transcribed spacer (ITS), β-tubulin (*tub2*), and translation elongation factor 1-α gene (*tef* 1-α), which led to the identification of a new host record, *Nigrospora macarangae*. This is the first *Nigrospora* species being recorded on *Ficus pumila*. Our findings significantly contribute to the understanding of the fungal diversity of *Ficus pumila* and provide valuable information on the species distribution of *Nigrospora*. We emphasize the need for future studies to comprehend the pathogenicity of these isolated species on *Ficus pumila*.

Keywords – Ascomycota – New host – Phylogeny – Taxonomy – Systematics

Introduction

Ficus pumila is a perennial vine belonging to *Moraceae* and is often widely used for ornamental purposes (Datiles & Acevedo-Rodríguez 2014). At the same time, its roots, stems, leaves, and fruits all have extremely high medicinal value, and it can also be used as a low-calorie health food with high edible value (Qi et al. 2021). However, studies on fungi associated with *Ficus pumila* are considerably less and there are only a few records on *Aschersonia*, *Cercospora*, *Fusarium*, *Glomerella*, *Sphaeropsis*, *Rhizoctonia*, *Phakopsora*, *Phellinus*, *Pestalotiopsis* and *Phytophthora* species (Farr & Rossman 2024). However, there is currently no existing research on *Nigrospora* species from *Ficus pumila*.

Nigrospora is an important ascomycetes genus with a worldwide distribution and a wide host range (Wang et al. 2017). The asexual morph of this genus is characterized by branched micronematous or semimacronematous conidiophores, monoblastic conidiogenous cells which are black, shiny and aseptate conidia. The sexual morph comprises perithecial ascomata, short-stalked

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asci with biseriate ascospores (Webster 1952, Wang et al. 2017, Raza et al. 2019). *Nigrospora* was introduced by Zimmerman (1902) to accommodate *N. panici*, which was isolated as an endophyte from *Panicum amphibium* leaves in Java, Indonesia (Uzor et al. 2015, Wang et al. 2017, Liu et al. 2024). Since then, *Nigrospora* has been recorded as a phytopathogen of many important economic crops. For example, *N. sphaerica* causes leaf blight on *Camellia sinensis* in China (Liu et al. 2015) and *N. musae* causes 'squirter' disease on bananas (Jones & Stover 2000). *Nigrospora* species are also considered a very interesting source of natural products due to their potential industrial applications (Chen et al. 2016). Currently, 49 *Nigrospora* species epithets are listed in Index Fungorum (2024).

In the present study, we observed the leaf spot symptoms associated with *Ficus pumila* vines in Guangdong province, China. We found that there are obvious lesions on the leaves of *Ficus pumila*, the middle of the lesions is yellowish brown, and the edges of the yellowish brown begin to turn yellow. The objectives of this study were to isolate and identify the fungus associated with *Ficus pumila* leaf spots based on multigene molecular phylogeny and morphology analysis.

Materials & Methods

Isolates and Morphology

Diseased leaves were collected from Dinghu Mountain, Guangzhou City, Guangdong Province, China. The diseased leaves were washed with water to remove dirt and dust. Then isolation is performed via the tissue isolation method (Senanayake et al. 2020). Leaves were cut into small pieces (5×5 mm) from the margin between the necrotic and healthy tissue of the diseased leaves. These small pieces were soaked for 1 min in 75% ethanol solution, soaked for 1 min in 2.5% sodium hypochlorite solution, and finally washed three times with sterilized distilled water for 1 min each time. The sterilized tissue fragments were dried on sterile filter paper and then transferred to potato dextrose agar (PDA). All PDA plates were cultured at a constant temperature of 25 ℃ for 2–4 days, and single hyphae were picked from the periphery of the grown colonies and inoculated on new PDA plates. After 2–3 times of purification pure cultures were obtained. All pure cultures obtained in this study are deposited in the culture collection of Zhongkai University of Agriculture and Engineering (ZHKUCC), China. Herbarium materials (as dry cultures) are deposited in the herbaria of Zhongkai University of Agriculture and Engineering (ZHKU), China.

For morphological characterization, 2–3 weeks old cultures growing on PDA were used. The mucilaginous mass was picked out under the Cnoptec sz650 stereomicroscope to make slides, and the spore morphology and structure were observed and recorded under the Nikon Eclipse 80i microscope (Nikon, Japan). Conidial length, width, and size of appendages were measured for 30 conidia per isolate using Tarosoft® Image Frame Works Version 0.9.7, and the mean values were calculated with their standard deviations (SDs). The pure cultures obtained in this study were all grown on PDA at 25 °C for one week, after 5 days the diameter of the cultures was measured and the colony morphology was recorded with a Nikon D300s digital camera (Nikon, Japan). Colony colours (top and bottom) are described by reference to the Rayner colour chart (Rayner et al. 1970).

DNA extraction and amplification

For genomic DNA extraction, mycelia were scraped from one-week-old cultures grown on the PDA. The total genomic DNA was extracted using MagPure Plant AS Kit (Magnetic bead hair plant DNA kit, China) following the manufacturer's instructions. Three genes were selected in this study: ITS, *tub2*, and *tef* 1-α were amplified using primer pairs ITS5/ITS4 (White et al. 1990), Bt-2F/Bt-4R (O'Donnell et al. 1997), and EF1-728F/EF-2 (O'Donnell & Cigelnik et al. 1998, Carbone et al. 1999).

The final Polymerase chain reaction (PCR) 25 μL consists of, 12.5 μL of 2×Taq Master Mix (Dye Plus) (mixture of FastTaq TM DNA Polymerase, buffer, dNTP Mixture, and stabilizer) (Beijing Qingke Biological Technology Co., Ltd., Beijing, PR China), 8.5 μL of ddH_2O, 1.5 μL of primers, and 1 μL of DNA template. PCR reactions were performed in a C1000 Touch TM thermal

cycler with the following amplification procedure: initial denaturation at 94 ℃ for 3 min, followed by 30 cycles consisting of denaturation at 94 ℃ for 30 s, annealing for 30 s and extension at 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. The annealing temperature of ITS and *tef* 1-α were 53 °C and the annealing temperature of *tub2* was 55 °C. After PCR amplification, a positive result XR+ (Bio-Rad, USA) was observed after 1% agarose gel electrophoresis under UV light using Gel DocTM XR Molecular Imager (Bio-Rad, USA). DNA sequencing was performed by Tianyi (Guangzhou, China) Co., Ltd. All sequence data generated in this study are deposited in NCBI Genbank.

Phylogenetic analysis

The sequencing chromatograms were examined using BioEdit version v.7.0.5.2 (Hall 1999), and low–quality regions were trimmed before sequence alignment. All sequences generated in this study were compared against using the National Center for Biotechnology Information (NCBI) search engine GenBank BLASTn for initial species identification. All reference sequences used in this study were downloaded from GenBank, which are listed in Table 1 (Chen et al. 2022). Alignments for each locus were generated using MAFFT v. 7 webserver (http://mafft.cbrc.jp/alignment/server (accessed 20 November 2022) and were manually improved using BioEdit v.7.0.5.2 (Hall 1999). For the final files were combined in ITS, *tub2* and *tef* 1–α order by BioEdit v.7.0.5.2 (Hall 1999). In the present study, maximum likelihood (ML) and Bayesian analysis (BI) were used in phylogenetic analyses.

The ML analysis was performed using RAxML–HPC2 on XSEDE version 8.2.8 (San Diego Supercomputer Center, CA, USA) (Stamatakis et al. 2008, 2014) in the CIPRES Science Gateway platform (Miller et al. 2010) using GTR+I+G model of evolution. For the ML analysis, ambiguous regions in the alignment were excluded, and gaps were treated as missing data. Tree stability was evaluated with 1,000 bootstrap replications. The BI analysis was performed in CIPRES (Miller et al. 2010) with MrBayes v. 3.2.7a (Huelsenbeck et al. 2001, Stamatakis et al.2014). The Markov chain Monte Carlo (MCMC) sampling approach was used to calculate posterior probabilities (PP) (Rannala & Yang 1996). Four simultaneous Markov chains were run for 10 million generations, and trees were sampled every 1,000th generation. From the 10,000 trees obtained, the first 2000, representing the burn-in phase, were discarded. To calculate posterior probabilities, the remaining 8000 trees were used. The phylogenetic trees were generated using Fig Tree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and edited using Adobe Illustrator 2018 (Adobe, USA).

Table 1 Strains of the *Nigrospora* species and related GenBank accession numbers of taxa included in this study.

Species	**Isolates**	**GenBank accession numbers**			**References**
		ITS	***tub2***	***tef* 1-α**	
Nigrospora aurantiaca	CGMCC 3.18130^{T}	KX986064	KY019465	KY019295	Wang et al. (2017)
N. aurantiaca	LC 12065	MN215771	MN329935	MN264010	Raza et al. (2019)
N. bambusae	CGMCC 3.18327^{T}	KY385307	KY385319	KY385313	Wang et al. (2017)
N. bambusae	LC 7244	KY385306	KY385320	KY385314	Wang et al. (2017)
N. brasiliensis	CMM 1214^{T}	KY569629	MK720816	MK753271	Crous et al. (2019)
N. brasiliensis	CMM 1217	KY569630	MK720817	MK753272	Crous et al. (2019)
N. camelliae-sinensis	CGMCC 3.18125^{T}	KX985986	KY019460	KY019293	Wang et al. (2017)
N. camelliae-sinensis	LC 13512	MN215775	MN329939	MN264014	Raza et al. (2019)
N. chinensis	CGMCC 3.18127^{T}	KX986023	KY019462	KY019422	Wang et al. (2017)
N. chinensis	LC 4660	KX986026	KY019548	KY019445	Wang et al. (2017)
N. cooperae	BRIP 72408b	OP035047	OP039537	OP039538	Lee et al. (2023)

Table 1 Continued

Species	Isolates	GenBank accession numbers			References
		ITS	*tub2*	*tef* 1-α	
N. cooperae	BRIP 72440a	OP035048	OP039540	OP039539	Lee et al. (2023)
N. covidalis	CGMCC 3.20538 = LC 4566^{T}	OK335209	OK431479	OK431485	Chen et al. (2022)
N. covidalis	LC 158337	OK335210	OK431480	OK431486	Chen et al. (2022)
N. endophytica	ARM973	OM265233	OP572420	OP572416	De Queiroz et al. (2022)
N. endophytica	ARM687	OM265226	OP572418	OP572415	De Queiroz et al. (2022)
N. falsivesicularis	CGMCC 3.19678^{T}	MN215778	MN329942	MN264017	Raza et al. (2019)
N. falsivesicularis	LC 13553	MN215779	MN329943	MN264018	Raza et al. (2019)
N. globosa	CGMCC 3.19633T	MK329121	MK336134	NA	Zhang et al. (2021)
N. globosa	LC 12441	MK329122	MK336135	NA	Zhang et al. (2021)
N. globospora	CGMCC 3.20539 = LC 8397^{T}	OK335211	OK431481	OK431487	Chen et al. (2022)
N. globospora	LC 15839	OK335212	OK431482	OK431488	Chen et al. (2022)
N. gorlenkoana	CBS 480.73^{T}	KX986048	KY019456	KY019420	Wang et al. (2017)
N. guilinensis	CGMCC 3.18124^{T}	KX985983	KY019459	KY019292	Wang et al. (2017)
N. guilinensis	LC 7301	KX986063	KY019608	KY019404	Wang et al. (2017)
N. hainanensis	CGMCC 3.18129T	KX986091	KY019464	KY019415	Wang et al. (2017)
N. hainanensis	LC 13514	MN215780	MN329944	MN264019	Raza et al. (2019)
N. lacticolonia	CGMCC 3.18123T	KX985978	KY019458	KY019291	Wang et al. (2017)
N. lacticolonia	LC 12060	MN215784	MN329948	MN264023	Raza et al. (2019)
N. macarangae	NCYUCC 19-0312	MW114320	NA	NA	Tennakoon et al. (2021)
N. macarangae	NCYUCC 19-0177	MW114319	NA	NA	Tennakoon et al. (2021)
N. macarangae	**ZHKUCC 23-0001**	**PP091033**	**PP646183**	**PP646180**	**This study**
N. macarangae	**ZHKUCC 23-0002**	**PP091034**	**PP646184**	**PP646181**	**This study**
N. macarangae	**ZHKUCC 23-0003**	**PP091035**	**PP646185**	**PP646182**	**This study**
N. macarangae	MFLUCC 19-0141^{T}	MW114318	NA	NA	Tennakoon et al. (2021)
N. magnoliae	LC 6704	KX986047	KY019571	KY019373	Wang et al. (2017)
N. magnoliae	MFLUCC 19-0112 = KUMCC 17-0246^{T}	MW285092	MW438334	NA	De Silva et al. (2021)
N. magnoliae	LC 6704	KX986047	KY019571	KY019373	Wang et al. (2017)
N. manihoticola	ARM645	OM265224	OM869479	OM914791	De Queiroz et al. (2022)
N. musae	CBS 319.34^{T}	KX986076	KY019455	KY019419	Wang et al. (2017)
N. musae	LC 6385	KX986042	KY019567	KY019371	Wang et al. (2017)
N. oryzae	LC 7293	KX985931	KY019601	KY019396	Wang et al. (2017)
N. oryzae	LC 2707	KX985954	KY019481	KY019307	Wang et al. (2017)
N. osmanthi	CGMCC 3.18126T	KX986010	KY019461	KY019421	Wang et al. (2017)
N. osmanthi	LC 4487	KX986017	KY019540	KY019438	Wang et al. (2017)
N. pernambucoensis	ARM974	OM265234	OM869481	OM914793	De Queiroz et al. (2022)

Table 1 Continued

Species	Isolates	GenBank accession numbers			References
		ITS	*tub2*	*tef* 1-α	
N. philosophiae-doctoris	CGMCC 3.20540 = LC 13398^{T}	OK335213	OK431483	OK431489	Chen et al. (2022)
N. philosophiae-doctoris	LC 15838	OK335214	OK431484	OK431490	Chen et al. (2022)
N. pyriformis	CGMCC 3.18122^{T}	KX985940	KY019457	KY019290	Wang et al. (2017)
N. pyriformis	LC 12075	MN215787	MN329988	MN264026	Raza et al. (2019)
N. rubi	CGMCC 3.18326^{T}	KX985948	KY019475	KY019302	Wang et al. (2017)
N. sacchari-officinarum	CGMCC 3.19335^{T}	MN215791	MN329954	MN264030	Raza et al. (2019)
N. sacchari-officinarum	LC 13531	MN215792	MN329955	MN264031	Raza et al. (2019)
N. saccharicola	CGMCC 3.19362^{T}	MN215788	MN329951	MN264027	Raza et al. (2019)
N. saccharicola	LC 12057	MN215789	MN329952	MN264028	Raza et al. (2019)
N. singulari	CGMCC 3.19334^{T}	MN215793	MN329956	MN264032	Raza et al. (2019)
N. singulari	LC 12068	MN215794	MN329957	MN264033	Raza et al. (2019)
N. sphaerica	LC 2840	KX985965	KY019492	KY019318	Wang et al. (2017)
N. sphaerica	LC 12083	MN215811	MN329974	MN264050	Raza et al. (2019)
N. vesicularifera	CGMCC 3.19333^{T}	MN215812	MN329975	MN264051	Raza et al. (2019)
N. vesicularifera	LC 12055	MN215814	MN329977	MN264053	Raza et al. (2019)
N. vesicularis	CGMCC 3.18128^{T}	KX986088	KY019463	KY019294	Wang et al. (2017)
N. vesicularis	LC 0322	KX985939	KY019467	KY019296	Wang et al. (2017)
N. zimmermanii	CBS 290.62^{T}	KY385309	KY385317	KY385311	Wang et al. (2017)
N. zimmermanii	LC 13534	MN215824	MN329987	MN264063	Raza et al. (2019)
Arthrinium obovatum	LC4940	KY494696	KY705166	KY705095	Wang et al. (2018)
A. malaysianum	CBS102053	NR120273	KF144988	KF145030	Crous et al. (2013)

^{T} Isolates represent ex-type. The isolates obtained in this study are bold.

Results

Phylogenetic analyses

Phylogenetic analyses were conducted using combined ITS, *tub2*, and *tef* 1-α sequence alignment of 63 *Nigrospora* strains (including type strains). *Arthrinium obovatum* (LC4940) and *A. malaysianum* (CBS 102053) were used as the outgroup taxon. The resulting maximum likelihood (ML) tree shared a similar topology with Bayesian analysis (BYPP). The best-scoring ML tree is shown in Fig. 1. The matrix had 908 distinct alignment patterns, with 26.91% of undetermined characters or gaps. Parameters for the GTR+I+G model of the combined ITS, *tub2* and *tef* 1-α were as follows: Estimated base frequencies; A = 0.216394, C = 0.302211, G = 0.241069, T = 0.240327; substitution rates AC = 1.052963, AG = 3.297901, AT = 1.241637, CG = 1.065654, CT = 5.015242, GT = 1.000000; gamma distribution shape parameter α = 0.933328. In the phylogenetic analysis, three isolates (ZHKUCC 23-0001, ZHKUCC 23-0002 and ZHKUCC 23-0003) from this study share a clade with other known species. To confirm the rigour of the taxa, rigorous morphological comparisons were performed. We identified these isolates as *Nigrospora macarangae*. Species description and illustration are given below.

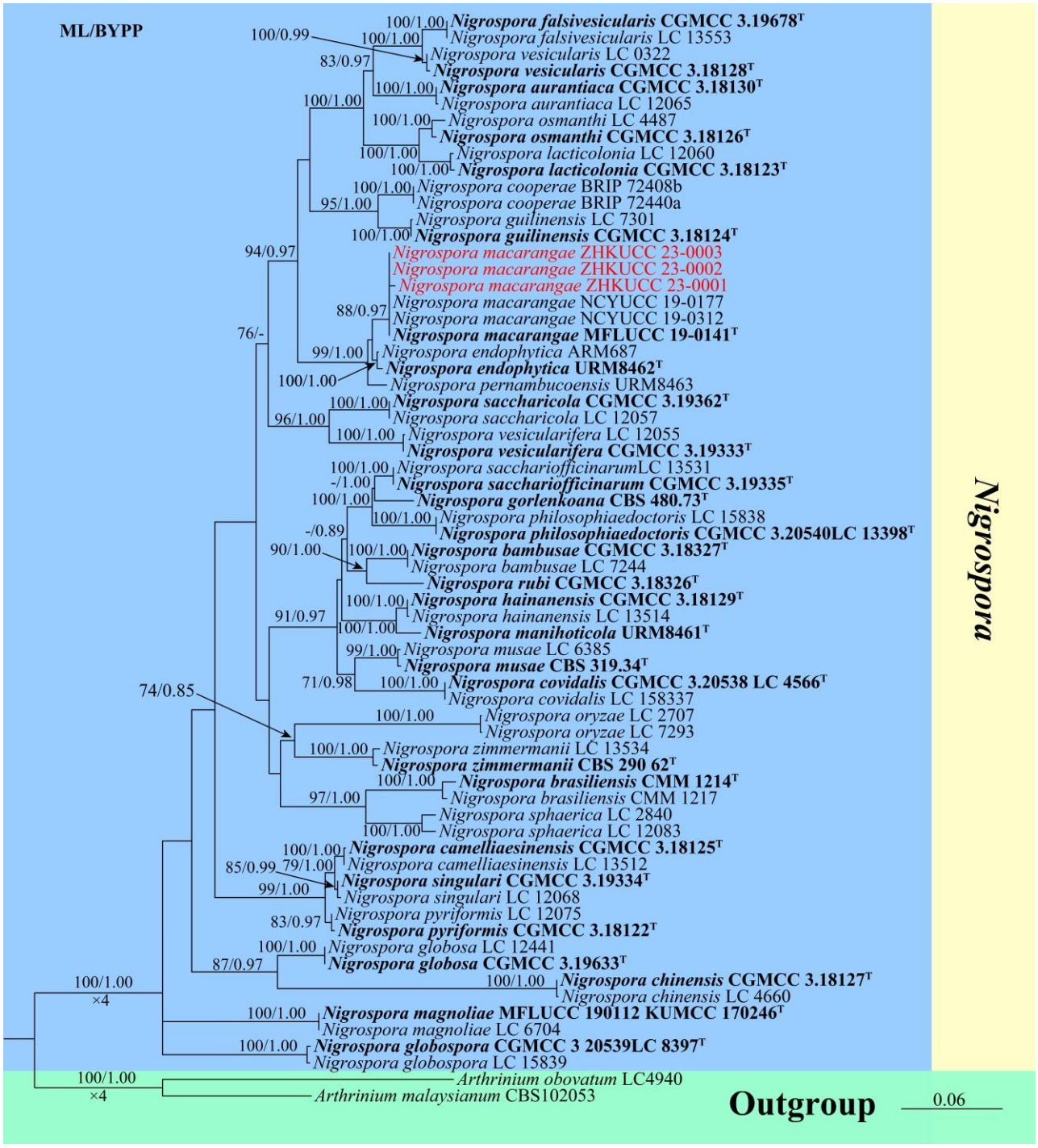

Fig. 1 – Phylogram generated from maximum likelihood (ML) analysis based on combined ITS, *tub2* and *tef* 1-α sequence alignment of *Nigrospora* species. The tree is rooted in *Arthrinium obovatum* (LC4940) and *A. malaysianum* (CBS102053). Maximum likelihood bootstrap support values ≥ 70% and Bayesian Inference posterior probabilities ≥ 0.90 (ML/ BYPP) are given at the nodes in common branches. The isolates obtained in this study are in red. T indicates ex-type strains. The scale bar represents the expected number of changes per site.

Taxonomy

Nigrospora macarangae Tennakoon, C.H. Kuo & K.D. Hyde, Fungal Diversity 108: 99 (2021) (Fig. 2)

Index Fungorum number: IF555454; Facesoffungi number: FoF09343

Associated with leaf spot on *Ficus pumila*. Sexual morph: Undetermined. Asexual morph on PDA: Conidia with mycelium grown on PDA. Conidiophores reduced to conidiogenous cells. Conidiogenous cell 4–8 × 5–15μm ($\bar{x}$= 5.3 × 9.8 μm, n = 25), transparent to dark brown, smooth to finely rough, subcylindrical to massive. Conidia 11–17 × 14–20 μm ($\bar{x}$ = 13.8 × 16.6 μm, n = 30) μm, immature stage light brown, some small oil droplet in the middle, transparent, solitary, spherical, nearly spherical, with smooth edges; mature stage, dark brown, a large oily drop in the middle, part of the back contains a distinct truncated basal scar, transparent, solitary, spherical, subspherical, smooth–walled.

Culture characteristics – Colonies on PDA reach 30–35 mm in diameter after 3 days. Colonies from above: medium dense, irregular, hypertrophic, slightly raised, smooth surface, scalloped, fluffy to velvety, smooth edge, off-white to brown edge, gray center; reverse: dark gray, with light gray edges and brown to black center.

Known distribution – Saprobic on dead leaves of *Macaranga tanarius* in Taiwan Province, China (Tennakoon et al. 2021).

Material examined – China, Guangdong Province, Guangzhou City, Dinghu Mountain, on *Ficus pumila*, 18 Nov 2021, Li Hua, living culture (ZHKUCC 23-0001, ZHKUCC 23-0002, ZHKUCC 23-0003).

Notes – In the phylogenetic analysis of the present study, three isolates from *Ficus pumila* clustered with *Nigrospora macarangae* with 88% ML bootstrap values and 0.97 BYPP values. (Fig. 1). Morphologically, our isolates share similar morphological characteristics with the type strain of *N. macarangae* (MFLUCC 19-0141) with few differences. The conidia size between our isolate (ZHKUCC 23-0001) and the type (MFLUCC 19-0141), 5.3 × 9.8 μm *vs*. 8.5 × 13 μm), has a sight different, however considering the large differences in the growth environments of the strains, we consider our strain as *N. macarangae*. This is the first record of *N. macarangae* associated with *Ficus pumila* leaf spot.

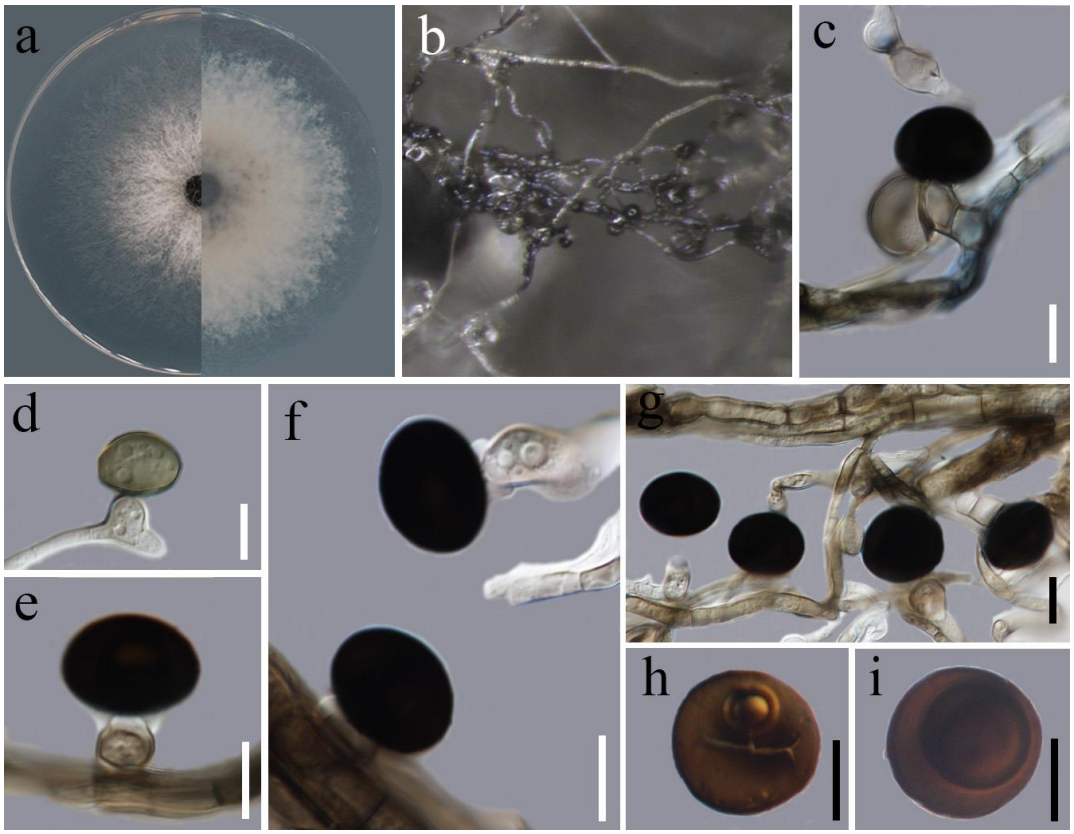

Fig. 2 – *Nigrospora macarangae* (ZHKUCC 23-0001, new host record). a Colonies from above and below (on PDA). b Hypae with conidial mass forming on culture. c–f Conidiogenous cells with conidia. g–i Conidia. Scale bars c–i = 10 μm.

Discussion

Through morphological and phylogenetic analysis, we identified a fungus isolated from *Ficus pumila* as *Nigrospora macarangae*. *Nigrospora* species are common pathogens associated with many hosts. *Nigrospora aurantiaca* has been found to infect tobacco and cause tobacco leaf spots (Huang et al. 2021), and *Nigrospora oryzae* has caused kiwifruit black spots (Li et al. 2018). *Nigrospora macarangae* was first isolated from the dead leaves of *Macaranga tanarius* (*Euphorbiaceae*) (Tennakoon et al. 2021). Therefore, this is the first report of *Nigrospora macaranga* on *Ficus pumila*. We isolated our strain as it is associated with leaf spots on *Ficus pumila*, which could be a potential pathogen. However, this needs to be further confirmed by follow-up pathogenicity essays. In the future, studying the fungal biodiversity of *Ficus pumila* and the identification of pathogenic fungi can significantly enhance *Ficus pumila* protection measures, consequently reducing maintenance costs.

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